

Molecular Mechanisms of Developmental Timing in *C. elegans* and *Drosophila* Review

Carl S. Thummel¹

Howard Hughes Medical Institute
Department of Human Genetics
15 North 2030 East Room 5100
University of Utah
Salt Lake City, Utah 84112

Characterization of the heterochronic genes has provided a strong foundation for understanding the molecular mechanisms of developmental timing in *C. elegans*. In apparent contrast, studies of developmental timing in *Drosophila* have demonstrated a central role for gene cascades triggered by the steroid hormone ecdysone. In this review, I survey the molecular mechanisms of developmental timing in *C. elegans* and *Drosophila* and outline how common regulatory pathways are beginning to emerge.

Detailed genetic and molecular studies have provided insights into the mechanisms by which two model organisms, *C. elegans* and *Drosophila*, control the timing of their development. At first glance, these pathways appear to be quite different. Genetic studies in *C. elegans* have revealed a network of heterochronic genes that control the timing of cell fate decisions during development. The heterochronic genes, however, exert no effect on the molting cycle, leaving it unclear how developmental transitions are regulated in *C. elegans*. In contrast, studies in *Drosophila* have defined a central role for the steroid hormone ecdysone in directing the major developmental transitions in its life cycle, including molting and metamorphosis. No heterochronic mutant phenotypes that alter the timing of cell fate decisions, however, have yet been described in flies.

Interestingly, studies over the past few years reveal a growing convergence in our understanding of the mechanisms of developmental timing in these two organisms. Several lines of evidence indicate that one or more cholesterol-derived hormones is required for molting in *C. elegans* and that this signal may act through members of the nuclear receptor superfamily—providing clear parallels with ecdysone signaling in *Drosophila*. In addition, homologs of *C. elegans* heterochronic genes have been identified in flies, and the induction of one of the key regulators in this pathway correlates with the ecdysone pulse that triggers the onset of *Drosophila* metamorphosis. Below, I review these recent advances and outline how future studies may unify our understanding of the mechanisms of developmental timing in these two model organisms.

Heterochronic Genes Direct Stage-Specific Cell Lineage Patterns in *C. elegans*

C. elegans develop into their adult form through a series of four larval stages, designated L1–L4, each punctuated by molting of the cuticle. Each larval stage is characterized by specific patterns of cell division, leading

to the formation of stage-specific cell types (Sulston and Horvitz, 1977). Heterochronic mutations were identified based on temporal transformations of these cell division patterns within epidermal lineages (Chalfie et al., 1981; Ambros and Horvitz, 1984). Below, I provide a brief overview of heterochronic gene function in *C. elegans*. Three recent reviews provide more detail on the genetics that underlie this model (Slack and Ruvkun, 1997; Ambros, 2000; Rougvie, 2001).

Loss-of-function *lin-14* mutations cause blast cells to skip the L1 stage and assume fates associated with L2, followed by normal development to the adult. In contrast, gain-of-function *lin-14* alleles lead to a reiteration of L1 cell fates at later developmental stages (Ambros and Horvitz, 1984). These phenotypes, combined with studies of a temperature-sensitive allele, indicate that LIN-14 activity is required for the L1 stage in development (Figure 1). Similarly, *lin-28* mutants indicate a role for this gene in L1/L2 stages to regulate L2/L3 cell fates. Loss-of-function *lin-28* mutants have no effect during L1 but cause premature expression of L3 fates during L2, while gain-of-function *lin-28* alleles cause cells to reiterate L2 fates at later larval stages (Ambros and Horvitz, 1984; Moss et al., 1997). *lin-14* and *lin-28* also depend on the wild-type activity of each other during L1, defining a positive feedback loop (Arasu et al., 1991; Moss et al., 1997) (Figure 1).

The recent identification of multiple loss-of-function *lin-41* alleles, combined with the effects of overexpressed wild-type LIN-41 protein, has defined this heterochronic gene as required for late larval stages (Slack et al., 2000). In *lin-41* mutants, lateral hypodermal cells develop normally through L1 and L2, but prematurely adopt an adult fate at the L3/L4 molt. Overexpression of wild-type LIN-41 results in the opposite phenotype, leading to a reiteration of the larval hypodermal cell fate at the end of L4 rather than the normal switch to adult development. The precocious heterochronic phenotype associated with *lin-41* mutants requires a wild-type copy of *lin-29*, placing *lin-29* function downstream from *lin-41* (Slack et al., 2000). *lin-29* mutants, in turn, display a later phenotype than those of *lin-41*, reiterating larval cell lineages at the adult stage (Ambros and Horvitz, 1984). This observation implies that *lin-29* is required for the larval-to-adult switch in the hypodermis. Taken together, genetic studies of these heterochronic genes position them in a temporal sequence that is required for proper epidermal cell fate decisions at each developmental stage, with *lin-14* required for L1/L2 fates, *lin-28* required for L2/L3 fates, *lin-41* required for L3/L4 fates, and *lin-29* required for adult cell fate (Slack and Ruvkun, 1997; Ambros, 2000; Slack et al., 2000) (Figure 1).

Negative Regulatory Interactions Determine the Temporal Order of Heterochronic Gene Function

Molecular studies of *lin-14*, *lin-28*, *lin-41*, and *lin-29* have both supported and extended the model derived from genetic analysis. Expression of LIN-14 and LIN-28 protein correlates with their proposed genetic functions.

¹ Correspondence: carl.thummel@genetics.utah.edu

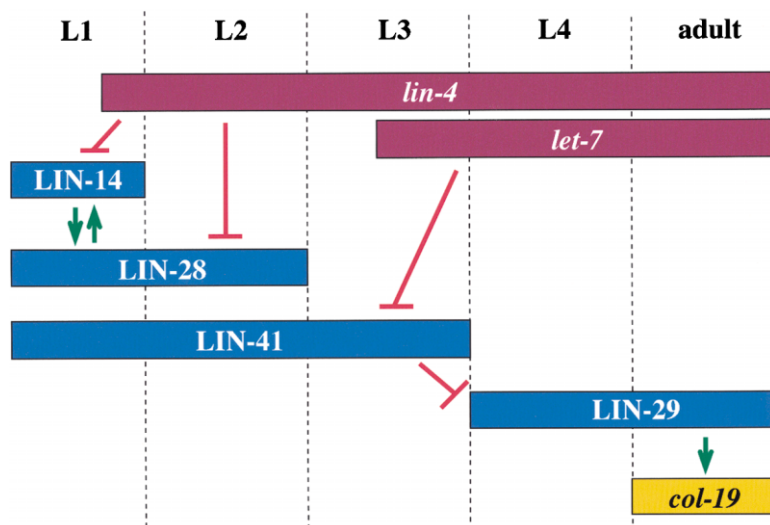


Figure 1. A Model for Heterochronic Gene Function during *C. elegans* Development

The four larval stages, L1–L4, are listed at top along with the adult stage of *C. elegans* development. Shown in the blue boxes below are the approximate times of protein expression for LIN-14, LIN-28, LIN-41, and LIN-29. Purple boxes represent the timing of *lin-4* and *let-7* small regulatory RNA expression and the orange box depicts the timing of the *col-19* adult cuticle gene. The lines and arrows indicate proposed direct regulatory interactions (Slack and Ruvkun, 1997; Ambros, 2000; Slack et al., 2000; Rougvie, 2001).

LIN-14 protein is first detected in late embryos, peaks in L1 larvae, and drops to low levels by the end of L1 (Ruvkun and Giusto, 1989). In *lin-14* gain-of-function mutants, LIN-14 protein persists beyond the L1 stage, correlating with the reiteration of L1 cell fate observed in this mutant background. Thus, the presence of LIN-14 protein defines the L1 stage of blast cell development. Similarly, LIN-28 protein peaks in late embryos and L1, remains detectable through L2, and is present at only very low levels during L3 (Moss et al., 1997). Gain-of-function *lin-28* mutations lead to stable expression of LIN-28 protein at later stages, again correlating with the observed changes in cell fate. These temporal gradients of expression thus fit with the functions of these genes predicted by genetic analysis.

In sharp contrast to the restricted temporal patterns of LIN-14 and LIN-28 protein accumulation, transcripts from these genes are present throughout development (Wightman et al., 1993; Moss et al., 1997). Consistent with this disparity between mRNA and protein accumulation, detailed studies have demonstrated that *lin-14* and *lin-28* mRNA are translationally controlled and have identified the *lin-4* heterochronic gene as a mediator of this regulation.

Genetic studies of *lin-4* indicate that it is a negative regulator of *lin-14* and *lin-28* (Ambros, 1989; Arasu et al., 1991; Wightman et al., 1991; Moss et al., 1997) (Figure 1). A *lin-4* null mutant phenocopies *lin-14* gain-of-function mutants and leads to persistent LIN-14 protein at later stages of development (Chalfie et al., 1981; Arasu et al., 1991). *lin-4* encodes two small RNAs, 61 and 22 nucleotides in length, with no apparent open reading frame (Lee et al., 1993). Gain-of-function *lin-14* mutations map to the 3'-untranslated region (3'-UTR) of the *lin-14* mRNA, a region with seven copies of a short sequence that could base pair with the *lin-4* RNAs (Wightman et al., 1991; Yeh, 1991). In addition, these 3' sequences are sufficient to confer appropriate temporal regulation on the translation of a heterologous gene, implicating these sites as direct targets for *lin-4* regulation (Wightman et al., 1993).

LIN-28 protein is also expressed at later stages in a *lin-4* mutant, suggesting that it may also be subject to

lin-4-dependent posttranscriptional regulation (Moss et al., 1997). The 3'-UTR of the *lin-28* mRNA contains a single potential *lin-4* binding site, and deletion of this sequence results in a gain-of-function *lin-28* mutant phenotype as well as persistent LIN-28 protein. Interestingly, the number of potential *lin-4* binding sites correlates with the timing of LIN-14 and LIN-28 expression. Thus, the seven potential binding sites in the *lin-14* mRNA may direct more rapid translational suppression of this target than the single site present in the *lin-28* mRNA (Slack and Ruvkun, 1997). Levels of *lin-4* expression also increase during L1 and remain high for the rest of development, suggesting that *lin-14* mRNA may be more sensitive to *lin-4* dose than is *lin-28* mRNA (Feinbaum and Ambros, 1999). The molecular mechanism by which *lin-4* suppresses LIN-14 and LIN-28 translation, however, and the mechanism that directs their distinct temporal profiles of protein accumulation, remain to be determined (Olsen and Ambros, 1999).

Remarkably, at least two other examples of negative posttranscriptional regulatory circuits have been identified among the heterochronic genes of *C. elegans*. Direct repressive interactions have been proposed between *let-7* and *lin-41* and between *lin-29* and *lin-41*, defining cell fates associated with later stages in the life cycle.

let-7 loss-of-function mutants recapitulate larval patterns of cell division at the end of L4 rather than executing the larval-to-adult switch, much like *lin-29* mutants (Reinhart et al., 2000). Overexpression of *let-7* from a transgenic array, in contrast, causes hypodermal cells to undergo premature terminal differentiation at the L3/L4 molt. Like *lin-4*, *let-7* directs the synthesis of a 21 nucleotide RNA that does not encode a protein product (Reinhart et al., 2000). Potential *let-7* binding sites can be found in the 3'-UTRs of five heterochronic genes. Functions for most of these sites remain to be defined. *lin-41*, however, appears to be a direct target for *let-7* regulation through *let-7* complementary sequences in its 3'-UTR (Reinhart et al., 2000). Consistent with this proposal, the timing of *let-7* induction correlates with down-regulation of LIN-41 protein in wild-type animals. Finally, genetic studies have demonstrated that improper regulation of *lin-41* can account for most of the lethal and

Table 1. *Drosophila* Homologs of *C. elegans* Heterochronic Genes

<i>C. elegans</i> Gene Product	Classification	<i>Drosophila</i> Homolog	BLAST Homologies	Reference
<i>lin-4</i>	regulatory RNA	none	none	Lee et al., 1993
<i>let-7</i>	regulatory RNA	<i>let-7</i>	100%	Pasquinelli et al., 2000
LIN-14	novel nuclear protein	none	none	Ruvkun and Giusto, 1989
LIN-28	cold shock domain, CCHC zinc fingers	CG17334	39%/148 aa (3)	Moss et al., 1997
LIN-29	C ₂ H ₂ zinc finger	CG10040 1(3)02102	75%/180 aa (3) 78%/150 aa (1)	Rougvie and Ambros, 1995
LIN-41	RBCC protein, NHL domain	CG1624	48%/192 aa (1)	Slack et al., 2000
LIN-42	PAS domain	Period	33%/109 aa (1)	Jeon et al., 1999
DAF-12	nuclear receptor	DHR96	64%/85 aa (1) DBD 25%/260 aa (5) LBD	Antebi et al., 2000

Heterochronic gene products discussed in the text are listed. *Drosophila* homologs predicted by the genome sequence have at least one EST in the databases. All *Drosophila* homologs, except the Period protein, gave a high BLAST score with the corresponding *C. elegans* protein when searching the *C. elegans* genome sequence. BLAST homologies are presented as percent identical amino acids over a given range of amino acids with the number of gaps in parentheses. DBD, DNA binding domain; LBD, ligand binding domain.

heterochronic phenotypes observed in *let-7* mutants, providing further evidence that *lin-41* is directly regulated by *let-7*. Thus, at least in the hypodermal cells, it appears that upregulation of the *let-7* RNA in L3 results in repression of *lin-41* translation, directing the proper timing of L3/L4 and L4/adult cell fates (Figure 1).

The precise temporal control of LIN-41 expression conferred by *let-7* is passed along to a downstream heterochronic gene, *lin-29* (Figure 1). Again, this may be mediated by direct stage-specific repression of translation. *lin-29* mRNA is first detected at L2 and remains high through later stages of development (Rougvie and Ambros, 1995; Bettinger et al., 1996). In contrast, LIN-29 protein does not appear in hypodermal cells until L4, after which it is maintained at high levels, correlating with the time of *lin-29* function in terminal differentiation of the adult cuticle. In a *lin-41* loss-of-function mutant, LIN-29 protein is prematurely expressed during L2 and L3 (Slack et al., 2000). This is consistent with genetic studies, mentioned above, that position *lin-29* downstream from *lin-41*. Moreover, aspects of the *let-7* mutant phenotype resemble *lin-29* loss-of-function phenotypes, consistent with indirect regulation of *lin-29* by *let-7* through *lin-41* (Reinhart et al., 2000) (Figure 1). *lin-41* encodes a member of the RBCC family of proteins, at least some of which bind directly to RNA (Slack et al., 2000). Thus, negative regulation of *lin-29* translation by *lin-41* could be imposed by a direct interaction with the LIN-41 protein. The function of LIN-41, however, and the mechanism by which it represses *lin-29* expression await further study.

The functions of LIN-14 and LIN-28 also remain to be defined. *lin-14* encodes a novel nuclear protein of unknown function and *lin-28* encodes a cytoplasmic protein with a cold shock domain and CCHC zinc finger motif (Table 1) (Ruvkun and Giusto, 1989; Moss et al., 1997; Hong et al., 2000). *lin-42*, a heterochronic gene that functions in early larval stages, encodes a PAS domain protein that is most similar to members of the PERIOD family of circadian rhythm regulators (Jeon et al., 1999). Consistent with this identity, the abundance of *lin-42* mRNA varies with a regular cycle. Rather than the standard 12 hr circadian cycle, however, the *lin-42* mRNA cycle is ~7 hr in length and is synchronized to

the molting cycle. There is no evidence for circadian control of this expression. The role of *lin-42* in the *C. elegans* heterochronic pathway thus awaits further characterization of its encoded protein. In contrast, another heterochronic gene, *daf-12*, encodes a member of the nuclear receptor superfamily, raising the intriguing possibility that heterochronic gene activity might be modulated by hormonal signaling (Antebi et al., 2000). Functions for *daf-12* are discussed in more detail below.

In conclusion, the heterochronic gene cascades that control developmental timing in *C. elegans* may involve at least three cases of negative posttranscriptional regulation. Two of these are conferred by small noncoding RNAs, *lin-4* and *let-7*. The third may be conferred by the LIN-41 RBCC protein. Taken together, these regulatory interactions define precise temporal gradients of LIN-14, LIN-28, LIN-41, and LIN-29 protein expression that, in turn, confer appropriate stage-specific cell fates during the *C. elegans* life cycle (Figure 1).

Temporal Boundaries and Temporal Identities Can Contribute to Developmental Timing

The elegance and relative simplicity of the heterochronic gene cascade has made it almost synonymous with developmental timing. Yet, the effects of the heterochronic mutations are limited to the patterns of cell division that occur at each developmental stage—few ties have been made to molting, the most overt manifestation of progression through the life cycle. The larval molts progress on schedule in heterochronic mutants, even though individual blast cells that contribute to the newly formed cuticle have the incorrect identity (Chalfie et al., 1981; Ambros and Horvitz, 1984). Timing of the cell division cycles is also unaffected by heterochronic mutations. The only heterochronic gene to show an effect on molting is *lin-29* which, when mutated, reiterates L4 fates, directing a series of supernumerary molts (Bettinger et al., 1996). Similarly, precocious activation of *lin-29* causes early termination of the molting cycle. These phenotypes, however, are most likely an indirect effect of the fate switch in *lin-29* mutants and not indicative of a direct role for this gene in the molt cycle. Thus, we are left with the question of how the temporal identity conferred by the heterochronic genes is related to the

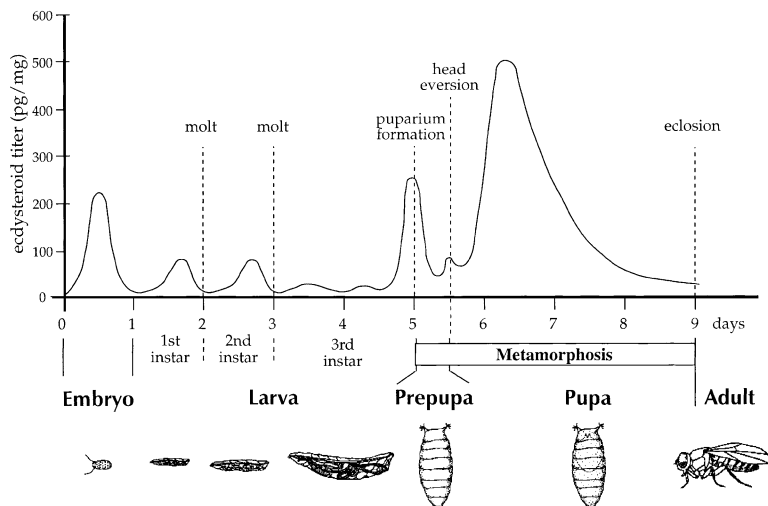


Figure 2. Ecdysone Pulses Trigger Each of the Major Developmental Transitions in *Drosophila*

The ecdysone titer profile is depicted as 20E equivalents in whole body homogenates (Riddiford, 1993). The major developmental transitions are marked by dotted lines.

major developmental landmarks that define the *C. elegans* life cycle.

An answer to this question can be achieved by returning to an analogy described by Ambros and Horvitz (1987) and later extended by Slack and Ruvkun (1997). These authors proposed that temporal patterning is analogous to spatial patterning, with the heterochronic genes conferring temporal identity much like the homeotic genes confer spatial identity. Extending this analogy one step further provides a foundation for comparing and contrasting the mechanisms of developmental timing in *C. elegans* and *Drosophila*. The specification of distinct spatial domains not only involves establishing segmental identity but also involves establishing boundaries between segments. For example, networks of segmentation genes define the segmental and parasegmental boundaries of the early *Drosophila* embryo, followed by the specification of segment identity by the homeotic genes. Indeed, without boundaries, no landmarks exist to allow each group of cells to acquire a distinct fate.

By analogy with spatial patterning, we can propose that temporal patterning also involves the establishment of both boundaries and identities. The *C. elegans* heterochronic genes can be thought of as temporal identity genes that define the timing of cell autonomous cell fate decisions during the life cycle. By analogy to the homeotic genes, the fact that the heterochronic genes have no effect on molting—the temporal boundaries of the *C. elegans* life cycle—is not surprising. Moreover, this classification posits the existence of a distinct set of temporal boundary genes that regulate transitions in the *C. elegans* life cycle. Indeed, recent genetic studies have identified several such genes in *C. elegans*—genes that are required for molting as well as progression through larval stages. Moreover, studies in *Drosophila* have identified temporal boundary genes that direct the major transitions in the life cycle in response to a systemic steroid signal. Below, I review the role of hormones in establishing temporal boundaries in *Drosophila* and propose that similar regulatory pathways may control progression through the *C. elegans* life cycle.

Ecdysone Pulses Define Temporal Boundaries in the *Drosophila* Life Cycle

Since the 1930s, detailed physiological, biological, genetic, and molecular studies have provided insights into

the mechanisms of developmental timing in *Drosophila*. Pulses of the steroid hormone ecdysone direct each of the major developmental transitions in the life cycle (Figure 2) (Riddiford, 1993). An ecdysone pulse during midembryonic development is of unknown function, although recent evidence indicates possible roles in morphogenesis and cuticle deposition (Chavez et al., 2000). Ecdysone pulses during the first and second larval instars trigger molting of the cuticle, a response that appears to be restricted largely to the epidermis (Riddiford, 1993). In contrast, a high titer ecdysone pulse at the end of the third larval instar triggers widespread changes throughout the animal, signaling the onset of prepupal development (Robertson, 1936). The larval salivary glands secrete a mixture of glue proteins that are used to affix the animal to a solid surface for puparium formation. The animal then shortens its body and the larval cuticle tans and hardens to form a protective pupal case. A subset of larval tissues, including the midgut, initiate programmed cell death, ridding the animal of obsolete tissues (Robertson, 1936). Meanwhile, the leg and wing imaginal discs evert and elongate to form rudiments of the adult appendages. A subsequent ecdysone pulse, ~12 hr after puparium formation, triggers the prepupal-pupal transition. In response to this signal, the head assumes its appropriate position by everting out from the anterior end of the puparium. This ecdysone pulse also triggers final elongation of the legs and wings as well as destruction of most remaining larval tissues, including the larval salivary glands (Robertson, 1936). These critical events form the basic body plan of the adult insect, with a head, thorax, abdomen, and appendages. Terminal differentiation then occurs over the following ~3.5 days of pupal development, followed by eclosion of the adult insect (Figure 2).

Prothoracicotropic Hormone Determines the Timing of Ecdysone Release

A neuropeptide called prothoracicotropic hormone (PTTH) is the key signal that initiates release of the precursor steroid ecdysone from the prothoracic glands (Nijhout, 1994; Henrich et al., 1999). Ecdysone is then converted by peripheral tissues into the physiologically active form of the hormone, 20-hydroxyecdysone (20E). PTTH has been purified from the silk moth, *Bombyx mori*, and

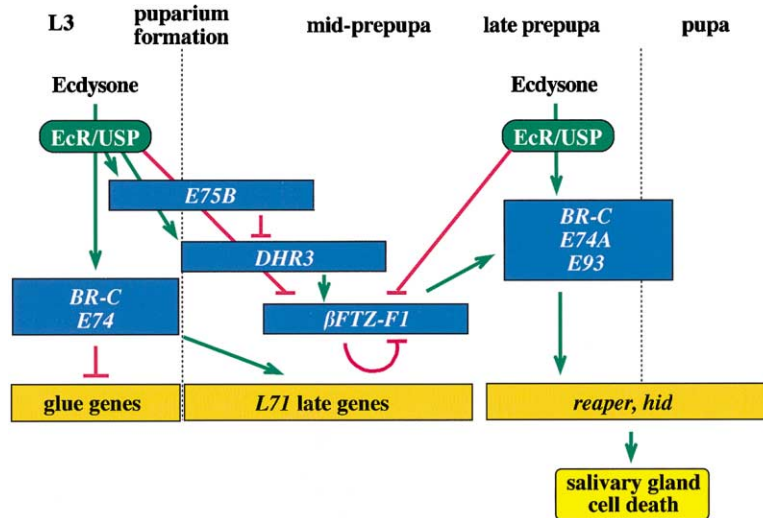


Figure 3. A Model for Ecdysone-Triggered Regulatory Interactions at the Onset of *Drosophila* Metamorphosis

This figure summarizes regulatory interactions described in the text. The blue boxes represent genes that encode ecdysone-inducible transcription factors. The orange boxes represent secondary response target genes. Green arrows represent inductive effects and red lines represent repressive effects.

the corresponding gene has been identified (Adachi-Yamada et al., 1994). Antibody stains have revealed that PTTH is synthesized in two large neurosecretory cells that reside in the dorsolateral region of each brain hemisphere (Mizoguchi et al., 1990). These neurons innervate the endocrine glands of the insect, providing direct transport of PTTH to its targets. The regulation of PTTH expression, however, and the signal for its release, remain unknown. The recent purification of a candidate PTTH from *Drosophila* raises the possibility of providing a better understanding of its regulation and function, although the identity of this factor remains unclear (Kim et al., 1997). The best match encoded by the *Drosophila* genome is an apparent integral membrane protein with EGF repeats (CG16882, T. Siegmund, personal communication). This protein is significantly larger than the protein detected by Kim et al. (1997) on Western blots and bears no similarity to known lepidopteran PTTHs. Further studies are thus required to determine whether this protein can indeed act as a PTTH in *Drosophila*.

It is interesting to note that the ecdysone pulses during *Drosophila* development occur with an approximate 12/24 hr cycle, defining the length of each developmental interval (Figure 2). Studies in the blood-feeding bug *Rhodnius prolixus*, which has been used extensively for characterization of PTTH action, indicate that PTTH and ecdysone release both exhibit pronounced circadian rhythmicity (Vafopoulou and Steel, 1996). Moreover, extensive data in a number of arthropods indicate that photoperiod is a key determinant of both the molting cycle and pupal diapause (Nijhout, 1994). For example, in the hornworm, *Manduca sexta*, PTTH release during a larval molt is gated by the photoperiod, providing a link between PTTH release and circadian signals (Truman, 1972). Only two neurons have been identified in *Drosophila* that innervate the prothoracic gland, and axons from the circadian pacemaker neurons project onto their dendritic fields, providing the means for direct circadian input to ecdysone release (Siegmund and Korge, 2001). Thus, although the regulation of the 12/24 hr ecdysone pulses in *Drosophila* remains to be determined, it seems likely that this cycle owes at least part of its timing to circadian control.

The Timing Conferred by Ecdysone Pulses Is Transduced through Stage-Specific Regulatory Cascades

Given the central role of ecdysone pulses in dictating progression through the fly life cycle, the question then arises of how the hormone exerts its effects at the molecular level. Extensive studies have been focused on the mechanisms of ecdysone action, and it is not the purpose of this review to summarize these pathways in detail. The reader is referred to several reviews on this topic (Thummel, 1996; Richards, 1997; Henrich et al., 1999; Riddiford et al., 2000). Rather, in keeping with the theme of developmental timing, I will focus on a key genetic circuit during late larval and prepupal development (Figure 3). This circuit illustrates how a repetitive systemic hormonal signal can be transduced into stage- and tissue-specific genetic and biological responses during development, highlighting the critical role of endocrine signaling in establishing temporal boundaries during the life cycle.

Responses to ecdysone are transduced by a heterodimer of two members of the nuclear receptor superfamily, the ecdysone receptor (EcR) and the RXR homolog Ultraspiracle (USP) (Koelle, 1992; Yao et al., 1992; Thomas et al., 1993). Like vertebrate nuclear receptors, the EcR/USP heterodimer functions as a ligand-dependent transcription factor, directly activating target gene transcription in response to the ecdysone signal. At least three protein isoforms are synthesized from the *EcR* locus, and the expression of these isoforms correlates with distinct cell- and tissue-specific responses to ecdysone during metamorphosis (Talbot et al., 1993).

Several primary response genes have been identified that are induced directly by the 20E/EcR/USP complex. These include three early regulatory genes—the *Broad-Complex* (BR-C), *E74*, and *E75*—defined originally as ecdysone-inducible puffs in the larval salivary gland polytene chromosomes (Ashburner et al., 1974). The BR-C encodes multiple zinc finger proteins (DiBello et al., 1991). *E74* encodes two proteins with an identical ETS DNA binding domain, designated *E74A* and *E74B* (Burtis et al., 1990), and *E75* encodes three members of the nuclear receptor superfamily, designated *E75A*, *E75B*, and *E75C* (Segraves and Hogness, 1990). The

E75 proteins are referred to as orphan nuclear receptors because no corresponding hormonal ligand has yet been identified for these factors.

The *BR-C* and *E74* are induced directly by both the late larval and prepupal ecdysone pulses in a wide range of target tissues (Boyd et al., 1991; Emery et al., 1994). Consistent with these broad patterns of expression, genetic studies have identified multiple biological functions for these genes at the onset of metamorphosis (Kiss et al., 1988; Restifo and White, 1992; Fletcher et al., 1995). Critical roles for the *BR-C* and *E74* in transducing both the late larval and prepupal ecdysone pulses can also be seen at the level of downstream secondary response gene expression. An ecdysone-induced switch in gene expression takes place in the salivary glands of late third instar larvae, in which the glue genes are repressed and the *L71* late puff genes are induced (Figure 3). This switch fails in *BR-C* mutants; the glue genes are not repressed and the *L71* genes are not induced (Guay and Guild, 1991). Similarly, the induction of the *L71* late genes is delayed and reduced in *E74A* mutants (Fletcher and Thummel, 1995a). The *BR-C* and *E74A* are later required for the induction of two key death-inducer genes in prepupal salivary glands, *reaper* and *head involution defective* (*hid*), consistent with the role of these two early genes in directing salivary gland cell death (Figure 3). Thus, the timing conferred by the late larval and prepupal ecdysone pulses is transduced by the *BR-C* and *E74*, resulting in appropriate patterns of target gene expression and, hence, appropriate biological responses. This observation, however, raises the question of how stage specificity is achieved. How is it that a repetitive hormonal signal can direct different biological responses at different times? Although the answer to this question is not yet complete, it is clear that stage-specific transcription factors play an important role in conferring this temporal specificity.

Cross-Regulation among Orphan Nuclear Receptors Directs Stage-Specific Responses to Ecdysone in *Drosophila*

E75B, *DHR3*, β *FTZ-F1* and *E93* are induced by ecdysone in a stage-specific manner at the onset of metamorphosis (Figure 3). *E75B*, *DHR3* and β *FTZ-F1* encode orphan members of the nuclear receptor superfamily (Segraves and Hogness, 1990; Koelle et al., 1992; Lavorgna et al., 1993). In contrast, *E93* encodes a novel nuclear protein that is required for larval salivary gland cell death (Baehracke and Thummel, 1995; Lee et al., 2000). Both *E75B* and *DHR3* are induced directly by the late larval ecdysone pulse, but their induction is also dependent on ecdysone-induced protein synthesis (Segraves and Hogness, 1990; Horner et al., 1995). This additional level of regulation leads to their accumulation at later times than true primary response gene products, such as *BR-C* and *E74A* (Figure 3). *DHR3* is both necessary and sufficient for the induction of β *FTZ-F1* in mid-prepupae and appears to exert this effect by binding to three adjacent sites in the β *FTZ-F1* promoter (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997; Lam et al., 1999). Interestingly, this inductive function of *DHR3* can be negatively regulated by heterodimerization with *E75B* (White et al., 1997). Thus, the timing of β *FTZ-F1* induction in mid-prepupae is determined by both induction

of the *DHR3* activator and decay of the *E75B* repressor (Figure 3). Ecdysone can also directly repress β *FTZ-F1* transcription, restricting its temporal window of expression to the mid-prepupal interval between the high titer late larval and prepupal pulses of ecdysone (Woodard et al., 1994). Finally, β *FTZ-F1* can repress its own transcription, ensuring that its expression will be of short duration (Figure 3).

Why is β *FTZ-F1* the target of such exquisite temporal control? Genetic studies have demonstrated that β *FTZ-F1* is an essential determinant of responses to the prepupal pulse of ecdysone. Loss-of-function β *FTZ-F1* mutants pupariate normally but die as prepupae and pupae with defects in adult head eversion and salivary gland cell death—changes that define the prepupal-pupal transition (Figure 4) (Broadus et al., 1999). These phenotypic effects are reflected by widespread effects on early ecdysone-induced gene expression in β *FTZ-F1* mutant prepupae, where the *BR-C*, *E74A*, *E75A*, and *E93* are all submaximally induced, while *EcR* and *usp* remain relatively unaffected. In addition, ectopic expression of β *FTZ-F1* in late third instar larvae results in enhanced ecdysone-induction of the *BR-C*, *E74A*, and *E75A*, as well as premature ecdysone induction of *E93*, switching the response to a prepupal pattern of early gene expression (Woodard et al., 1994). These observations suggest that β *FTZ-F1* provides competence for genetic responses to the prepupal ecdysone pulse, directing the appropriate stage-specific developmental responses that comprise the prepupal-pupal transition (Broadus et al., 1999).

The *BR-C* and *E74A* therefore play multiple roles in transducing ecdysone pulses, acting in different pathways at the larval-prepupal and prepupal-pupal transitions (Figure 3). In contrast, *DHR3*, *E75B*, and β *FTZ-F1* exert a stage-specific function during the onset of metamorphosis, defining the proper outcome of the prepupal ecdysone pulse. The induction of these orphan nuclear receptors in early and mid-prepupae indicates that puparium formation has been achieved and that responses to the next ecdysone pulse should be distinct from responses to the last ecdysone pulse. In this manner, cross-regulation between *E75B*, *DHR3*, and β *FTZ-F1* ensures that development progresses forward in response to the repetitive hormonal signal, refining this signal into distinct stage-specific biological pathways.

In closing, it is interesting to note that not all effects of ecdysone proceed through transcriptional control by the *EcR/USP* heterodimer. A recent study reports that, as in mammals, ecdysone can act through a nongenomic mechanism (independently of transcription and translation) to regulate the proliferation of neural precursor cells in the optic lobes of *Manduca* (Champlin and Truman, 2000). This discovery opens new possibilities for coordinating biological responses to ecdysone, providing a more rapid and direct means by which the steroid can affect cellular activity. It will be interesting to see if nongenomic mechanisms can account for other responses to ecdysone during development.

Endocrine Signaling Establishes Temporal Boundaries

The predominant lethal phenotype caused by a decrease in ecdysone titer, or by mutations in *EcR* or *usp*,

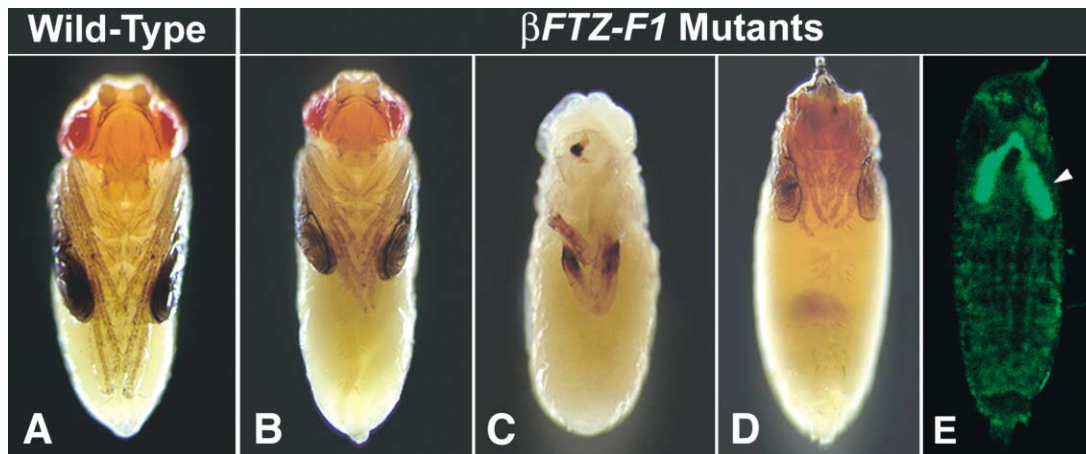


Figure 4. $\beta FTZ-F1$ Is Required for the Prepupal-Pupal Transition during *Drosophila* Metamorphosis

Shown are a control wild-type pharate adult just prior to eclosion (A) and $\beta FTZ-F1$ mutants that die as a pharate adult (B), early pupa (C), or cryptocephalic pharate adult (D), all with short distorted legs. (E) Confocal image of persistent larval salivary glands expressing GFP in a cryptocephalic $\beta FTZ-F1$ mutant pupa ~ 10 hr after the salivary glands are normally destroyed. Adapted from Figure 4 of Broadus et al. (1999). Reproduced with permission.

is developmental arrest at the major life cycle transitions—at larval molts, puparium formation, the prepupal-pupal transition, or pupal development (Sliter and Gilbert, 1992; Bender et al., 1997; Hall and Thummel, 1998; Schubiger et al., 1998; Li and Bender, 2000). Thus, ecdysone pulses establish the duration of each developmental stage, defining the temporal boundaries in the fly life cycle. As a corollary to this, the genes that encode the ecdysone receptor heterodimer, *EcR* and *usp*, can be classified as temporal boundary genes. These genes transduce the timing conferred by ecdysone pulses to dictate appropriate progression through the life cycle. In contrast, *DHR3* and $\beta FTZ-F1$ act as temporal identity genes during the onset of metamorphosis, distinguishing responses to the prepupal ecdysone pulse from those triggered by the previous pulse of ecdysone at puparium formation. *E93* is also a candidate for a temporal identity gene. It is selectively expressed during metamorphosis in a spatial pattern that foreshadows larval midgut and salivary gland cell death and is both necessary and sufficient for a death response (Baehrecke and Thummel, 1995; Lee et al., 2000). *E93* mutants, however, also display phenotypes during metamorphosis that appear unrelated to cell death, including defects in puparium formation and early pupal lethality. Further analysis of these phenotypes is required in order to clarify the potential role of *E93* in conferring temporal identity.

Do temporal identity genes exist in *Drosophila* that specify cell identity at one stage in the life cycle, like the heterochronic genes of *C. elegans*? Mutations in the *Drosophila anachronism* gene have been proposed to result in a heterochronic phenotype (Ebens et al., 1993). The precocious development of neuroblasts in *anachronism* mutants, however, is due to premature release from an arrest in proliferation rather than a temporal switch in cell identity. Several more clear examples of heterochrony have been reported in *Drosophila*, as described below, although these can be attributed to defects in the establishment of temporal boundaries rather

than the direct assignment of temporal identity. It thus remains unclear whether precise switches in cell identity similar to those seen in the *C. elegans* heterochronic mutants will be found in *Drosophila*, or whether this phenotype is restricted to organisms that rely on a fixed cell lineage for their development. One intriguing possibility, as discussed below, is that mutational analysis of *Drosophila* homologs of *C. elegans* heterochronic genes will uncover an evolutionarily conserved temporal identity pathway. Alternatively, it is possible that, by analogy with spatial patterning during embryogenesis, temporal specificity could be achieved through combinatorial interactions among nonstage-specific ecdysone-inducible regulatory genes, such as *BR-C* and *E74*. A similar model has been proposed by D. Hogness to explain how overlapping combinations of ecdysone-inducible transcription factors could direct distinct tissue-specific responses to ecdysone, termed the tissue coordination model (Thummel et al., 1990). The available evidence indicates that this may be a critical means of achieving both spatial and temporal specificity in ecdysone responses during the fly life cycle (Talbot et al., 1993; Fletcher and Thummel, 1995b).

It is important to realize that the use of systemic lipophilic hormones to confer temporal boundaries is not restricted to insects. Frogs also undergo a dramatic metamorphosis during their life cycle, in which the body plan is massively remodeled from a swimming tadpole to a reproductively active adult form. Like insects, this transition is entirely dependent on a systemic hormone (thyroid hormone), its corresponding nuclear receptor (thyroid hormone receptor/RXR heterodimer), and functions through the activation of stage- and tissue-specific gene cascades (Brown et al., 1995; Shi, 1996). Similar hormone-dependent developmental transitions occur during mammalian development, including steroid-triggered sexual maturation in humans. Indeed, systemic endocrine signaling provides an ideal means of coordinating tissue development and directing orderly progression through

the life cycle. Given this function, the logical question arises as to whether *C. elegans*, and nematodes in general, might utilize an endocrine system to establish temporal boundaries in their life cycle. Several lines of evidence, described below, lead to the conclusion that this is likely to be the case.

Failure to Establish Precise Temporal Boundaries Can Lead to Heterochronic Phenotypes in *Drosophila*

Defects in hormone signaling can lead to heterochronic phenotypes in insects. The classic example of this is the sesquiterpenoid juvenile hormone (JH) which, upon application to the cuticle of a late stage larva, results in the local deposition of a new larval cuticle while the rest of the animal deposits an adult cuticle in response to ecdysone signaling (Wigglesworth, 1958). Extensive studies in *Rhodnius* and lepidopteran insects have supported a role for JH in determining the nature of an ecdysone-triggered molt (Riddiford, 1996). Unfortunately, however, this response is not seen in the larvae of higher Diptera such as *Drosophila*, and thus little is known about the molecular mechanisms by which JH can control the developmental outcome of an ecdysone pulse. The recent demonstration that *BR-C* expression can be controlled by JH, combined with the identification of JH-inducible genes in *Drosophila*, provides future directions for defining the molecular mechanisms of JH action (Dubrovsky et al., 2000; Zhou and Riddiford, 2001).

An ecdysone deficiency in *Drosophila* also leads to a remarkable heterochronic phenotype in which second instar larvae appear to directly pupariate without progressing through a third larval instar. This phenotype has been seen in *dre4* mutants and IP3 receptor mutants, both of which display a reduction in ecdysone titer (Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997). One possible explanation for this phenotype is that these animals are not responding to the reduced ecdysone pulse during the second larval instar and thus missing the cue that would normally trigger a second-to-third instar larval molt. The higher titer ecdysone pulses associated with metamorphosis could then trigger the next developmental transition which, interestingly, is puparium formation, resulting in a prolonged second instar and the apparent absence of a third instar larval stage.

The converse of this phenotype has also been seen in *Drosophila*, once again due to a defect in ecdysone signaling. While the larval midgut and imaginal discs attempt to initiate metamorphosis in *usp* mutant third instar larvae, the epidermis deposits a supernumerary cuticle, recapitulating aspects of an earlier genetic program (Hall and Thummel, 1998).

Hence, precise temporal coordination during development appears to require efficient transduction of each ecdysone pulse through the EcR/USP heterodimer. In this sense, these heterochronic phenotypes in *Drosophila* are distinct from those seen in the heterochronic mutants in *C. elegans*. These phenotypes indicate that clear and definite boundaries need to be established in order for groups of cells to acquire their appropriate identity through the onset of metamorphosis. If the boundaries are not clear, then some cells may escape temporal coordination and acquire an inappropriate identity relative to the rest of the organism.

Steroid Hormones May Establish Temporal Boundaries in the *C. elegans* Life Cycle through Nuclear Receptor Signaling

Larval molting in *C. elegans* and *Drosophila* display many features in common (Singh and Sulston, 1978; Riddiford, 1993), reflecting their common evolutionary link as members of the Ecdysozoa clade (Aguinaldo et al., 1997). In *C. elegans*, as in *Drosophila*, each new cuticle is characterized by a unique combination of stage-specific cuticle proteins (Liu et al., 1995; Charles et al., 1998). Understanding how these genes are regulated in *C. elegans* provides one avenue to approach the mechanisms by which the molt is controlled. This may be particularly fruitful given that the heterochronic genes determine the identity of epidermal cells at each molt and the *col-19* adult cuticle gene is a direct target of *lin-29* regulation (Liu et al., 1995).

Perhaps most significant is the recent demonstration that cholesterol is required for molting in *C. elegans*. Like *Drosophila*, *C. elegans* is unable to synthesize cholesterol and thus requires a constant dietary source of the steroid (Chitwood, 1992). Transfer of wild-type *C. elegans* to cholesterol deficient medium has no effect on the first generation of animals, most likely due to their utilization of stored cholesterol (Yochem et al., 1999). However, a small proportion of larvae in the second generation (~5%) display defects in shedding their old cuticle during a molt (Figure 5A). This observation is consistent with earlier studies that have shown that sterol starvation of other nematodes leads to incomplete molting (Bottjer et al., 1985; Coggins et al., 1985). Interestingly, a similar molting defect is seen in *lrp-1* mutants, which arrest development primarily at the L3/L4 transition (Figures 5B and 5C) (Yochem et al., 1999). Many of these mutants survive for several days stuck within their old cuticle, providing a remarkable similarity to the larval molting defects associated with reduced ecdysone signaling in *Drosophila*. *lrp-1* encodes a gp330/megalin-related protein that is a member of the low density lipoprotein receptor family (Yochem et al., 1999). This gene identity is consistent with a number of different mechanisms for LRP-1 action. Given the similarity between sterol starvation and the *lrp-1* mutant phenotypes, however, an intriguing possibility is that *lrp-1* is required for the endocytosis of dietary cholesterol, which, in turn, could act as an essential precursor for a molting hormone.

A closer parallel between molting in nematodes and *Drosophila* is provided by parasitic nematodes, which appear to use ecdysteroids as a molting hormone. Ecdysteroids can be detected in the larvae of several parasitic nematode species, and larval molting can be stimulated by nanomolar concentrations of ecdysteroids in two intestinal parasites, *Nematospirides dubius* and *Ascaris suum* (see references in Sluder and Maina, 2001). Orthologs of the *EcR* and *usp* genes have also been identified in two parasitic nematodes, *D. immitis* and *B. malayi*, although no transcriptional response to a hormone has yet been demonstrated in these species (Sluder and Maina, 2001). In contrast, the *C. elegans* genome does not encode EcR or USP orthologs, and ecdysteroids have not been detected in this organism. Nonetheless, the requirement for cholesterol as well as the roles of nuclear receptors in larval molting described below, support the proposal that one or more steroid hormones

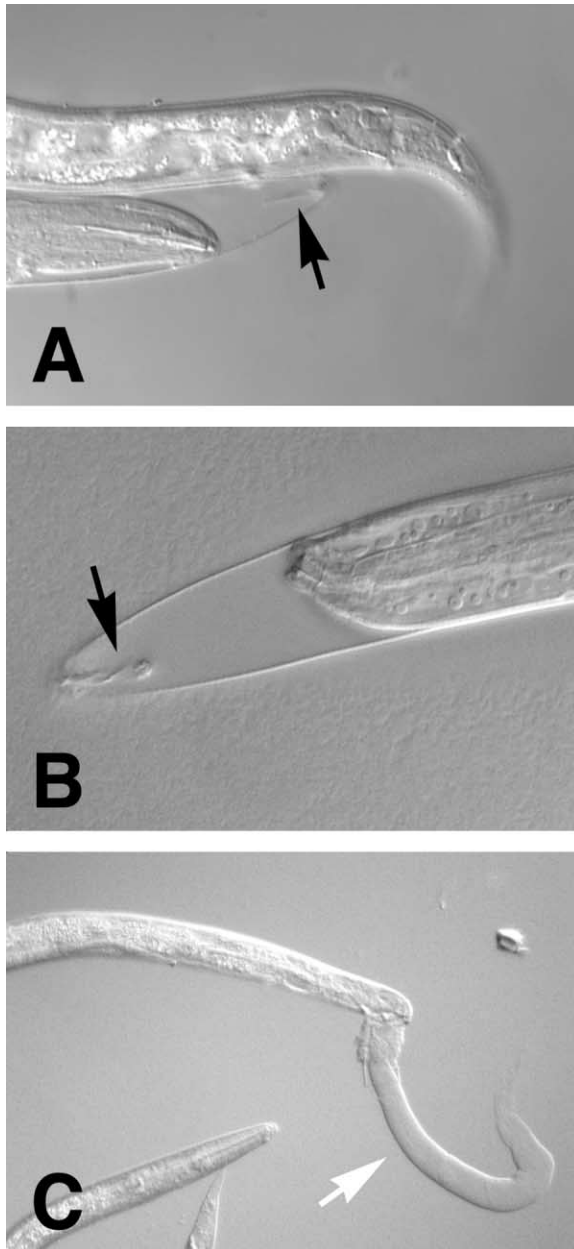


Figure 5. Molting Defects in *C. elegans*

(A) A wild-type L3 larva maintained on medium deficient in sterols with a displaced anterior and pharyngeal cuticle (black arrow).

(B) An *lrp-1* mutant L2 larva with attached pharyngeal cuticle (black arrow).

(C) An old cuticle remains attached to the end of an *lrp-1* mutant L3 larva (white arrow).

Adapted from Figure 4 of Yochem et al. (1999). Reproduced with permission.

is involved in the regulation of the *C. elegans* life cycle. Interestingly, the *C. elegans* genome encodes 270 members of the nuclear receptor superfamily, significantly more than the 21 nuclear receptors encoded by the *Drosophila* genome and 48 known vertebrate nuclear receptor superfamily members (Sluder and Maina, 2001). Further functional studies of these genes as well as biochemical characterization of organic extracts prepared from staged

C. elegans larvae should provide insights into the mechanisms by which steroids could regulate the molting cycle in this organism.

The *C. elegans* Orthologs of DHR3 and FTZ-F1 Are Required for Larval Molting

Only a few genes have been identified in *C. elegans* that could establish temporal boundaries by regulating larval molting. Remarkably, these genes encode the LRP-1 gp330/megalin-related protein described above as well as two members of the nuclear receptor superfamily: NHR-23 (also known as CHR3) and NHR-25. Although this sample size is small, the implied link between cholesterol derivatives and nuclear receptor activity is striking.

nhr-23 is widely expressed during the first half of *C. elegans* embryogenesis and then becomes more restricted to the epidermis through the rest of development (Kostrouchova et al., 1998). Interference in *nhr-23* activity by RNAi leads to a small proportion of animals that die during embryogenesis with defects in elongation and morphogenesis (Kostrouchova et al., 1998). The majority of surviving larvae display molting defects and arrest development at the L3 or L4 stage. Application of dsRNA at different times in the life cycle leads to defects at each molt, indicating a requirement for *nhr-23* at each stage in the life cycle (Kostrouchova et al., 2001). The levels of *nhr-23* mRNA appear to fluctuate with the molting cycle, with peak expression immediately preceding each molt and lowest levels at the molt (Kostrouchova et al., 2001). Interestingly, this rhythmic pattern of expression is similar to that of at least some *C. elegans* cuticle genes, implying a possible regulatory connection (Johnstone and Barry, 1996). This modulation is also reminiscent of the peaks in early gene expression and cuticle gene expression that precede the larval molts in *Drosophila*, raising the question of whether a hormone might confer this timing in *C. elegans* (Thummel et al., 1990; Talbot et al., 1993; Charles et al., 1998).

Like *nhr-23*, *nhr-25* is expressed throughout the *C. elegans* life cycle, primarily in the epidermis, although more detailed time courses through larval stages have not yet been reported (Asahina et al., 2000; Gissendanner and Sluder, 2000). Most progeny of wild-type hermaphrodites injected with *nhr-25* dsRNA die as embryos with morphogenetic defects. The surviving larvae arrest their development at early stages and display defects in molting. A similar embryonic lethal phenotype is seen in homozygous *nhr-25* mutants that carry a deletion within the gene (Asahina et al., 2000). This embryonic lethality could be due to defects in the integrity of the hypodermis as well as more direct effects on midembryonic movements. The similarity between the phenotype of *disembodied* mutations in *Drosophila*, which disrupt a cytochrome P450 required for steroidogenesis, and the *nhr-23* and *nhr-25* embryonic lethal phenotypes provide an interesting connection (Chavez et al., 2000). The significance of this similarity, however, requires a more definitive understanding of ecdysone functions during *Drosophila* embryonic development as well as the roles of *nhr-23* and *nhr-25* in morphogenesis.

The known regulatory link between *DHR3* and *FTZ-F1*, the *Drosophila* orthologs of *nhr-23* and *nhr-25*, raises

the question of whether this link has been conserved through evolution. No effect on *nhr-25* expression, however, was seen in animals treated with *nhr-23* dsRNA, although these experiments resulted in only an ~ 4 -fold decrease in *nhr-23* expression and, in part, depended on a *nhr25::gfp* construct to monitor the effects on *nhr-25* expression (Kostrouchova et al., 2001). It would be interesting to repeat these experiments using a *nhr-23* null mutant and following the levels of endogenous *nhr-25* promoter activity. In contrast, a clear evolutionary connection can be made between the functions of *nhr-25* and *FTZ-F1*, both of which are required for larval molting during development (Asahina et al., 2000; Gissendanner and Sluder, 2000; Yamada et al., 2000). Thus, not only is evidence accumulating that *C. elegans* may regulate progression through its life cycle by means of hormone signaling, but the possibility also exists that this signal could be transduced by members of the nuclear receptor superfamily, providing a clear parallel with the mechanisms of ecdysone action in *Drosophila*.

daf-12* Provides a Tie between Hormone Signaling and Heterochronic Gene Function in *C. elegans

A third nuclear receptor superfamily member, encoded by the *daf-12* locus, provides an important link between possible hormone signaling and the heterochronic pathway in *C. elegans*. *daf-12* is a complex genetic locus that regulates multiple pathways during development (Antebi et al., 1998). Null mutations in *daf-12* define an essential role in dauer larva formation, the diapause state of *C. elegans* that allows survival through periods of starvation. Genetic epistasis studies have shown that *daf-12* functions at the end of the dauer signaling pathway, downstream from the major environmental and physiological signals that act through TGF β and insulin pathways to control dauer larva formation. Some *daf-12* mutant alleles also display highly penetrant heterochronic phenotypes, with *daf-12* controlling L3 developmental events. Hence, this gene provides a critical link between the heterochronic genes and the dauer larva alternative during development. It is interesting to note that diapause is controlled by hormones in all insects studied, raising the possibility that *daf-12* might direct dauer formation through hormonal input (Nijhout, 1994; Antebi et al., 1998).

The identity of *daf-12* as a member of the nuclear receptor superfamily is consistent with this model (Antebi et al., 2000). In addition, *daf-12* mutations that result in penetrant heterochronic phenotypes map to the region that functions as a ligand binding domain in hormone-regulated nuclear receptors, implying possible hormonal input into the heterochronic functions of this gene. Better evidence for hormonal regulation of *daf-12* activity, however, arises from analysis of the *daf-9* gene in *C. elegans*. Epistasis studies position *daf-9* immediately upstream of *daf-12* and demonstrate that all signaling for dauer larva formation goes through *daf-9* to *daf-12* (D. Riddle and A. Antebi, personal communication). Remarkably, *daf-9* encodes a cytochrome P450 that is related to known vertebrate steroidogenic P450s. Although the role of *daf-9* in steroidogenesis remains to be shown, this gene identity and the epistatic relationship between *daf-9* and *daf-12* strongly argue in support

of ligand regulation of *daf-12* activity during development. Genetic analysis of other *C. elegans* P450s as well as further studies of *daf-9* should provide more insight into this pathway.

The *Drosophila let-7* Homolog May Be Induced by Ecdysone

A recent study has shown that the *let-7* heterochronic regulatory RNA has been conserved in a wide range of species, including vertebrate, ascidian, hemichordate, mollusc, annelid, and arthropod (Pasquinelli et al., 2000). Interestingly, the temporal control of *let-7* also appears to be conserved, with expression correlating with the onset of late developmental stages. Most intriguing from the perspective of this review, *let-7* induction could be triggered by a known developmental timer—rising at the end of the third larval instar in *Drosophila* in apparent synchrony with the ecdysone pulse, and maintained at high levels throughout pupal development. More precise time points, as well as analysis of RNA from organs cultured in the presence of 20E, will be required to determine if *let-7* is induced directly by ecdysone in flies. Several potential binding sites for the EcR/USP heterodimer map within 1–2 kb of the *Drosophila let-7* gene, providing support for a direct regulatory connection (A. Bashirullah, personal communication). The *Drosophila* genome also contains a gene that is similar to the *let-7* target gene, *lin-41* (CG1624, Table 1), with potential *let-7* binding sites in its 3'-UTR (A. Bashirullah, personal communication). In addition, the *Drosophila* homolog of *daf-12*, *DHR96*, has a potential *let-7* binding site in its 3'-UTR, although the function of *DHR96* remains to be established (K. King-Jones, personal communication). Homologs of LIN-28 and LIN-29 are also encoded by the fly genome (Table 1). The discovery of these *Drosophila* genes, along with the potential ecdysone induction of fly *let-7*, provides exciting directions for future research. It will be interesting to determine if these genes have conserved their temporal identity functions from *C. elegans* to *Drosophila*.

Future Directions for Timing

The apparent differences in our understanding of developmental timing in *C. elegans* and *Drosophila* are a direct consequence of the different experimental approaches that were used in these organisms. Forward genetic screens in *C. elegans* led to identification of the heterochronic genes and their roles in conferring temporal identity, while the absence of screens for lethal mutants with molting defects left a gap in our understanding of how temporal boundaries are established in this organism. In contrast, the rich history of endocrinology in *Drosophila* led to the identification of hormone-triggered gene cascades that define temporal boundaries, while little understanding has been achieved regarding how temporal identity might be conferred in flies. It is clear that the complementarity in our understanding of developmental timing mechanisms in *C. elegans* and *Drosophila* provides a strong foundation that can guide further experimentation in both organisms, and that this work should lead to a more unified understanding of how timing is controlled during the development of higher organisms.

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References

- Adachi-Yamada, T., Iwani, M., Kataoka, H., Suzuki, A., and Ishizaki, H. (1994). Structure and expression of the gene for the prothoracicotrophic hormone of the silkworm *Bombyx mori*. *Eur. J. Biochem.* 22, 633–643.
- Aguinaldo, A.M., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., Raff, R.A., and Lake, J.A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* 387, 489–493.
- Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in *C. elegans*. *Cell* 57, 49–57.
- Ambros, V. (2000). Control of developmental timing in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* 10, 428–433.
- Ambros, V., and Horvitz, H.R. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* 226, 409–416.
- Ambros, V., and Horvitz, H.R. (1987). The *lin-14* locus of *Caenorhabditis elegans* controls the time of expression of specific postembryonic developmental events. *Genes Dev.* 1, 398–414.
- Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. *Development* 125, 1191–1205.
- Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes Dev.* 14, 1512–1527.
- Arasu, P., Wightman, B., and Ruvkun, G. (1991). Temporal regulation of *lin-14* by the antagonistic action of two other heterochronic genes, *lin-4* and *lin-28*. *Genes Dev.* 5, 1825–1833.
- Asahina, M., Ishihara, T., Jindra, M., Kohara, Y., Katsura, I., and Hirose, S. (2000). The conserved nuclear receptor Ftz-F1 is required for embryogenesis, moulting and reproduction in *Caenorhabditis elegans*. *Genes Cells* 5, 711–723.
- Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb. Symp. Quant. Biol.* 38, 655–662.
- Baehrecke, E.H., and Thummel, C.S. (1995). The *Drosophila* *E93* gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. *Dev. Biol.* 171, 85–97.
- Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., and Hogness, D.S. (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* 91, 777–788.
- Bettinger, J.C., Lee, K., and Rougvie, A.E. (1996). Stage-specific accumulation of the terminal differentiation factor LIN-29 during *Caenorhabditis elegans* development. *Development* 122, 2517–2527.
- Bottjer, K.P., Weinstein, P.P., and Thompson, M.J. (1985). Effects of an azasteroid on growth, development and reproduction of the free-living nematodes *Caenorhabditis briggsae* and *Panagrellus redivivus*. *Comp. Biochem. Physiol.* 82, 99–106.
- Boyd, L., O'Toole, E., and Thummel, C.S. (1991). Patterns of *E74A* RNA and protein expression at the onset of metamorphosis in *Drosophila*. *Development* 112, 981–995.
- Broadus, J., McCabe, J.R., Endrizzi, B., Thummel, C.S., and Woodward, C.T. (1999). The *Drosophila* β FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. *Mol. Cell* 3, 143–149.
- Brown, D.D., Wang, Z., Kanamori, A., Eliceiri, B., Furlow, J.D., and Schwartzman, R. (1995). Amphibian metamorphosis: a complex program of gene expression changes controlled by the thyroid hormone. *Recent Prog. Horm. Res.* 50, 309–315.
- Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell* 61, 85–99.
- Chalfie, M., Horvitz, H.R., and Sulston, J.E. (1981). Mutations that lead to reiterations in the cell lineages of *C. elegans*. *Cell* 24, 59–69.
- Champlin, D.T., and Truman, J.W. (2000). Ecdysteroid coordinates optic lobe neurogenesis via a nitric oxide signaling pathway. *Development* 127, 3543–3551.
- Charles, J.-P., Chihara, C., Nejad, S., and Riddiford, L.M. (1998). Identification of proteins and developmental expression of RNAs encoded by the 65A cuticle protein gene cluster in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 28, 131–138.
- Chavez, V.M., Marques, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Natze, J.E., and O'Connor, M.B. (2000). The *Drosophila* *disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development* 127, 4115–4126.
- Chitwood, D. (1992). Nematode sterol biochemistry. In *Physiology and Biochemistry of Sterols*, G. Patterson and W. Nes, eds. (Champaign, IL: American Oil Chemist's Society), pp. 257–293.
- Coggins, J.R., Schaefer, F.W., 3rd, and Weinstein, P.P. (1985). Ultrastructural analysis of pathologic lesions in sterol-deficient *Nippostrongylus brasiliensis* larvae. *J. Invertebr. Pathol.* 45, 288–297.
- DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., and Guild, G.M. (1991). The *Drosophila* *Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* 129, 385–397.
- Dubrovsky, E.B., Dubrovskaya, V.A., Bilderback, A.L., and Berger, E.M. (2000). The isolation of two juvenile hormone-inducible genes in *Drosophila melanogaster*. *Dev. Biol.* 224, 486–495.
- Ebens, A.J., Garren, H., Cheyette, B.N., and Zipursky, S.L. (1993). The *Drosophila* *anachronism* locus: a glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell* 74, 15–27.
- Emery, I.F., Bedian, V., and Guild, G.M. (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* 120, 3275–3287.
- Feinbaum, R., and Ambros, V. (1999). The timing of *lin-4* RNA accumulation controls the timing of postembryonic developmental events in *Caenorhabditis elegans*. *Dev. Biol.* 210, 87–95.
- Fletcher, J.C., and Thummel, C.S. (1995a). The *Drosophila* *E74* gene is required for the proper stage- and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. *Development* 121, 1411–1421.
- Fletcher, J.C., and Thummel, C.S. (1995b). The ecdysone-inducible *Broad-Complex* and *E74* early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. *Genetics* 141, 1025–1035.
- Fletcher, J.C., Burtis, K.C., Hogness, D.S., and Thummel, C.S. (1995). The *Drosophila* *E74* gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development* 121, 1455–1465.
- Gissendanner, C.R., and Sluder, A.E. (2000). *nhr-25*, the *Caenorhabditis elegans* ortholog of *ftz-f1*, is required for epidermal and somatic gonad development. *Dev. Biol.* 221, 259–272.
- Guay, P.S., and Guild, G.M. (1991). The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. *Genetics* 129, 169–175.
- Hall, B.L., and Thummel, C.S. (1998). The RXR homolog Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development* 125, 4709–4717.
- Henrich, V., Rybczynski, R., and Gilbert, L.I. (1999). Peptide hormones, steroid hormones, and puffs: Mechanisms and models in insect development. *Vitam. Horm.* 55, 73–125.
- Hong, Y., Lee, R.C., and Ambros, V. (2000). Structure and function analysis of LIN-14, a temporal regulator of postembryonic developmental events in *Caenorhabditis elegans*. *Mol. Cell. Biol.* 20, 2285–2295.

- Horner, M., Chen, T., and Thummel, C.S. (1995). Ecdysone regulation and DNA binding properties of *Drosophila* nuclear hormone receptor superfamily members. *Dev. Biol.* 168, 490–502.
- Jeon, M., Gardner, H.F., Miller, E.A., Deshler, J., and Rougvie, A.E. (1999). Similarity of the *C. elegans* developmental timing protein LIN-42 to circadian rhythm proteins. *Science* 286, 1141–1146.
- Johnstone, I.L., and Barry, J.D. (1996). Temporal reiteration of a precise gene expression pattern during nematode development. *EMBO J.* 15, 3633–3639.
- Kageyama, Y., Masuda, S., Hirose, S., and Ueda, H. (1997). Temporal regulation of the mid-prepupal gene *FTZ-F1*: DHR3 early late gene product is one of the plural positive regulators. *Genes Cells* 2, 559–569.
- Kim, A.J., Cha, G.H., Kim, K., Gilbert, L.I., and Lee, C.C. (1997). Purification and characterization of the prothoracicotropic hormone of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 94, 1130–1135.
- Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., and Fristrom, J.W. (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* 118, 247–259.
- Koelle, M.R. (1992). Molecular analysis of the *Drosophila* ecdysone receptor complex. PhD thesis, Stanford University, Stanford, California.
- Koelle, M.R., Segraves, W.A., and Hogness, D.S. (1992). DHR3: a *Drosophila* steroid receptor homolog. *Proc. Natl. Acad. Sci. USA* 89, 6167–6171.
- Kostrouchova, M., Krause, M., Kostrouch, Z., and Rall, J.E. (1998). CHR3: a *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. *Development* 125, 1617–1626.
- Kostrouchova, M., Krause, M., Kostrouch, Z., and Rall, J.E. (2001). Nuclear hormone receptor CHR3 is a critical regulator of all four larval molts of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 98, 7360–7365.
- Lam, G.T., Jiang, C., and Thummel, C.S. (1997). Coordination of larval and prepupal gene expression by the DHR3 orphan receptor during *Drosophila* metamorphosis. *Development* 124, 1757–1769.
- Lam, G., Hall, B.L., Bender, M., and Thummel, C.S. (1999). DHR3 is required for the prepupal-pupal transition and differentiation of adult structures during *Drosophila* metamorphosis. *Dev. Biol.* 212, 204–216.
- Lavorgna, G., Karim, F.D., Thummel, C.S., and Wu, C. (1993). Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl. Acad. Sci. USA* 90, 3004–3008.
- Lee, C.Y., Wendel, D.P., Reid, P., Lam, G., Thummel, C.S., and Baehrecke, E.H. (2000). E93 directs steroid-triggered programmed cell death in *Drosophila*. *Mol. Cell* 6, 433–443.
- Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75, 843–854.
- Li, T.-R., and Bender, M. (2000). A conditional rescue system reveals essential functions for the ecdysone receptor (*EcR*) gene during molting and metamorphosis in *Drosophila*. *Development* 127, 2897–2905.
- Liu, Z., Kirch, S., and Ambros, V. (1995). The *Caenorhabditis elegans* heterochronic gene pathway controls stage-specific transcription of collagen genes. *Development* 121, 2471–2478.
- Mizoguchi, A., Oka, T., Kataoka, H., Nagasawa, H., Suzuki, A., and Ishizaki, H. (1990). Immunohistochemical localization of prothoracicotropic hormone-producing cells in the brain of *Bombyx mori*. *Dev. Growth Differ.* 32, 591–598.
- Moss, E.G., Lee, R.C., and Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the *lin-4* RNA. *Cell* 88, 637–646.
- Nijhout, H. (1994). *Insect Hormones* (Princeton, NJ: Princeton University Press).
- Olsen, P.H., and Ambros, V. (1999). The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* 216, 671–680.
- Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P., et al. (2000). Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature* 408, 86–89.
- Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901–906.
- Restifo, L.L., and White, K. (1992). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in *Drosophila*: salivary glands, muscle, and gut. *Roux's Arch. Dev. Biol.* 201, 221–234.
- Richards, G. (1997). The ecdysone regulatory cascades in *Drosophila*. *Adv. Dev. Biol.* 5, 81–135.
- Riddiford, L.M. (1993). Hormones and *Drosophila* Development. In *The Development of Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 899–939.
- Riddiford, L. (1996). Molecular aspects of juvenile hormone action in insect metamorphosis. In *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, L. Gilbert, J. Tata, and B. Atkinson, eds. (New York: Academic Press), pp. 223–251.
- Riddiford, L.M., Cherbas, P., and Truman, J.W. (2000). Ecdysone receptors and their biological actions. *Vitam. Horm.* 60, 1–73.
- Robertson, C.W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principal morphological changes. *J. Morphol.* 59, 351–399.
- Rougvie, A.E. (2001). Control of developmental timing in animals. *Nat. Gen. Rev.* 2, 690–701.
- Rougvie, A.E., and Ambros, V. (1995). The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. *Development* 121, 2491–2500.
- Ruvkun, G., and Giusto, J. (1989). The *Caenorhabditis elegans* heterochronic gene *lin-14* encodes a nuclear protein that forms a temporal developmental switch. *Nature* 338, 313–319.
- Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., and Bender, M. (1998). *Drosophila* EcR-B ecdysone receptor isoforms are required for larval molting and neuron remodeling during metamorphosis. *Development* 125, 2053–2062.
- Segraves, W.A., and Hogness, D.S. (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* 4, 204–219.
- Shi, Y.-B. (1996). Thyroid hormone-regulated early and late genes during amphibian metamorphosis. In *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, eds. (New York: Academic Press), pp. 505–538.
- Siegmund, T., and Korge, G. (2001). Innervation of the ring gland of *Drosophila melanogaster*. *J. Comp. Neurol.* 431, 481–491.
- Singh, R., and Sulston, J.E. (1978). Some observations on molting in *Caenorhabditis elegans*. *Nematologica* 24, 63–71.
- Slack, F., and Ruvkun, G. (1997). Temporal pattern formation by heterochronic genes. *Annu. Rev. Genet.* 31, 611–634.
- Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. (2000). The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. *Mol. Cell* 5, 659–669.
- Sliter, T.J., and Gilbert, L.I. (1992). Developmental arrest and ecdysteroid deficiency resulting from mutations at the *dre4* locus of *Drosophila*. *Genetics* 130, 555–568.
- Sluder, A.E., and Maina, C.V. (2001). Nuclear receptors in nematodes: themes and variations. *Trends Genet.* 17, 206–213.
- Sulston, J.E., and Horvitz, H.R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 56, 110–156.

- Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* 73, 1323–1337.
- Thomas, H.E., Stunnenberg, H.G., and Stewart, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* 362, 471–475.
- Thummel, C.S. (1996). Flies on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* 12, 306–310.
- Thummel, C.S., Burtis, K.C., and Hogness, D.S. (1990). Spatial and temporal patterns of *E74* transcription during *Drosophila* development. *Cell* 61, 101–111.
- Truman, J.W. (1972). Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworms. *J. Exp. Biol.* 57, 805–820.
- Vafopoulou, X., and Steel, C.G. (1996). The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: Re-evaluation of its role in development. *Proc. Natl. Acad. Sci. USA* 93, 3368–3372.
- Venkatesh, K., and Hasan, G. (1997). Disruption of the IP₃ receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr. Biol.* 7, 500–509.
- White, K.P., Hurban, P., Watanabe, T., and Hogness, D.S. (1997). Coordination of *Drosophila* metamorphosis by two ecdysone-induced nuclear receptors. *Science* 276, 114–117.
- Wigglesworth, V. (1958). Some methods for assaying extracts of the juvenile hormone in insects. *J. Insect Phys.* 2, 73–84.
- Wightman, B., Burglin, T.R., Gatto, J., Arasu, P., and Ruvkun, G. (1991). Negative regulatory sequences in the *lin-14* 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev.* 5, 1813–1824.
- Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* 75, 855–862.
- Woodard, C.T., Baehrecke, E.H., and Thummel, C.S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* 79, 607–615.
- Yamada, M., Murata, T., Hirose, S., Lavorgna, G., Suzuki, E., and Ueda, H. (2000). Temporally restricted expression of transcription factor β FTZ-F1: significance for embryogenesis, molting and metamorphosis in *Drosophila melanogaster*. *Development* 127, 5083–5092.
- Yao, T., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* 71, 63–72.
- Yeh, W.H. (1991). Genes acting late in the signaling pathway for *Caenorhabditis elegans* dauer larval development. PhD thesis, University of Missouri-Columbia.
- Yochem, J., Tuck, S., Greenwald, I., and Han, M. (1999). A gp330/megalin-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. *Development* 126, 597–606.
- Zhou, B., and Riddiford, L.M. (2001). Hormonal regulation and patterning of the broad-complex in the epidermis and wing discs of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* 231, 125–137.